Marine Natural Products.
I. The Search for *Aplysia* Terpenoids in Red Algae

BY

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A NUMBER OF REPORTS exist (SOMMER *et al.*, 1937; WINKLER, 1959; ALLEN & DAWSON, 1960; PRESCOTT & JAHNES, 1962) from which one might deduce that the natural product content of marine mollusks and algae are related. There have been reports of phenolic substances (SAITO & NAKAMURA, 1951), terpenes (KATAYAMA, 1962), and organic bromides (MAUTNER *et al.*, 1953; AUGIER & MASTAGLI, 1956), all or part of which might be responsible for the antibiotic properties of algae and indirectly for similar properties in marine fauna. It will be interesting to determine whether or not these compounds found in algae are absorbed unchanged by the animals that feed on them.

The isolation and structure determination of substances from *Aplysia kurodai* (BABA) (YAMAMURA & HIRATA, 1963) does provide reason for speculation. The compounds are bromo-sesquiterpenes which might easily have been the result of feeding on plants containing phenolic bromides, or the actual terpenes, since the amount found varies with the time of collection. Another report by WINKLER (1961) suggests that *Aplysia californica* (COOPER) may concentrate toxic substances in the digestive gland.

An investigation of benthic algae may provide evidence for the suspected relationship between *Aplysia* and its foodstuffs as well as provide more conclusive insight into the nature of the antibiotics so often reported. Since the isolation of the bromides from *Aplysia* represents one of the few reported naturally occurring organic bromides, further study would be of interest from many viewpoints.

Reports on the feeding habits of the two animals by SAITO & NAKAMURA (1961) and WINKLER (1959) indicate that red algae are preferred although green algae are sometimes eaten. An investigation of the red algae is perhaps the most interesting for several reasons. First, this group contains a great variety of forms. Secondly, the greatest variety of plant sterols has been isolated from red algae (MILLER, 1962). It is also the group in which the occurrence of bromides, phenols, and terpenes has been reported.

One of the red algae which *Aplysia californica* has been observed to prefer (WINKLER & DAWSON, 1963) is *Plocamium pacificum* (KYLIN). As this plant is readily available in the Los Angeles area, we began our studies on it.

METHODS

The plant was collected at low tide, washed in fresh water and cleaned by hand to insure the removal of animal matter. The cleaned plants were ground with a minimal amount of water and ice in a blender and the pulp extracted with acetone. The acetone extraction was carried out in a large glass flask fitted with a Hershberg stirrer and into which acetone was distilled from a pot. The overflow returned to the same pot thereby concentrating the extract. The concentrate was removed frequently until all of the extractable matter had been obtained. Acetone was removed and the aqueous layer extracted with ether. The ether extracts were combined, dried over magnesium sulfate and concentrated through a fractionation column. The dark green semicrystalline residue was chromatographed on 60-100 mesh Florex, eluting with hexane, benzene, ether and methanol in mixtures of increasing polarity.

Thin layer chromatography was carried out with standard 40 x 200 mm plates. The plates were prepared with the Desaga/Brinkman model S-11 adjustable applicator with a nominal thickness of 0.25 mm according to the

instructions supplied with the equipment. The stationary phase was silica gel G. The plates were dried in an oven and stored over calcium chloride. The elution solvents were of reagent grade and were used without further purification. The solvent was allowed to run 10 cm from the point of compound application up to a carefully cleared area to insure a uniform solvent front. Development of finished chromatograms was accomplished with a chromogenic reagent of 10% sulfuric acid in water containing 0.1% ammonium molybdate and then heating to about 250° C.

The melting points are uncorrected. Spectral determinations were carried out on a Perkin-Elmer Infracord (sodium chloride optics), a Cary UV Recording Spectrometer and a Varian 60 mc Nuclear Magnetic Spectrometer.

RESULTS AND DISCUSSION

From 100 g (dry weight) of *Plocamium pacificum* (Kylin) was obtained 9.3 g of a dark green residue, smelling of tide pools. The oil was chromatographed on 500 g of Florex, and the following major fractions were obtained (see Table 1).

The first major fraction eluted appeared to be homogeneous from thin layer chromatography (TLC). An Rf of 0.77 was obtained with hexane. Recrystallization from ethyl ether was difficult and the gelatinous waxy solid appeared to melt at about 40° C. An infrared spectrum showed only bands associated with saturated hydrocarbons. This is presumably one of the high molecular weight hydrocarbons associated with most plants.

The second major fraction when analyzed by TLC using 25% ethyl ether in benzene, gave two major components: a white crystalline portion, 0.25 g, Rf 0.50, and a yellow oil, 0.35 g, Rf 0.95, with an odor like that of the major fraction.

Recrystallization of the solid fraction from ethanol, as platelets, or from methanol, as needles, gave a homogeneous product as shown by TLC. The melting point was 141.5 - 143.5 and a mixture melting point with purified cholesterol gave no depression. A Liebermann-Burchard test was also positive and identical with cholesterol. Furthermore the nuclear magnetic resonance spectrum as well as the infrared spectrum were identical with those of cholesterol. This confirms earlier reports by Tsuda (1957) of cholesterol in Pacific red algae.

The yellow oil appears to be of chemical interest since spectral studies indicate the presence of a saturated ketone. The infrared absorption is low (1753 cm^{-1}), indicating the possibility of a ketone in a 5-membered ring. The ultraviolet absorption shows a maximum at 250 mμ in 95% ethanol and an analysis of the NMR spectra gives a well defined split methyl adsorption (1.30δ), methylene adsorption, vinyl adsorption (5.3δ) and low field adsorption (7.5δ).

The bright red crystals are presumably a carotenoid. The absorption maxima in carbon disulfide are 550, 516, and 452 mμ.

Before chlorophyll began to bleed from the column the fourth fraction was collected. The light yellow oil was insoluble in carbon tetrachloride and difficultly soluble in chloroform. Its spectral properties indicated a saturated ketone. Reaction with carbonyl reagents confirmed this.

When the chlorophyll began to run through, the column was washed with 10% methyl alcohol in ethyl ether. The resultant dark oil was saponified by refluxing under a nitrogen atmosphere, in a methanolic potassium hydroxide solution. The neutral fraction obtained from an ether extraction of a basic aqueous suspension was clear, since all of the color remained in the aqueous phase. Concentration of the ether extract gave a gum which showed little evidence of functionality on spectral analysis.

None of the fractions or the original extract showed the presence of a phenol as evidenced also by a negative ferric chloride test. Although halogen appeared to have been present in the original extract no evidence for it was found in the individual fractions. This absence is perhaps not unexpected because of the diversity of red algae in the Pacific.

Table 1

	Solvent	Weight	Characteristics
1	Hexane	0.37 g	White waxy solid
2	Hexane-benzene	0.62 g	Yellow, semicrystalline, odor of tide pools
3	Benzene	0.040 g	Bright red crystals
4	Benzene-ethyl ether	0.174 g	Yellow oil
5	Ethyl ether-methanol	—	Dark green gum

Of interest is the report by BERGMAN (1960) that cholesterol is only a minor sterol of invertebrates. Perhaps the red algae may be the source of that sterol when found in some organisms. Undoubtedly the cholesterol content of some algae may render them unfit as a human food source.

The ketone fractions may serve as precursors to the animal products. This would require that the animal have some metabolic pathway capable of brominating the organic intermediate. It is perhaps more likely that we shall find plant products more closely related to those from the animal. Halogen fixation is an uncommon occurrence in living organisms, therefore the disclosure of the source of organic bromides will be interesting.

SUMMARY

Chemical relationships between marine organisms, for example that between *Gonyaulax* and shellfish, may be quite common. The antibiotic activity attributed to marine plants and animals may stem from the same source.

In typical benthic red algae, we have not detected phenols or organic bromides, which might be expected as direct precursors to products found in *Aplysia*. The several ketonic fractions isolated require further study to determine if they are structurally related to the animal products. The presence of cholesterol as a major sterol in red algae has been confirmed.

ACKNOWLEDGMENT

This investigation was supported in part by a Public Health Service fellowship I-FI-GM-24, 965-01 from the National Institute of General Medical Sciences. We also wish to thank Miss Kathleen Matsomoto for her assistance on this project.

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